JOURNAL OF BACTERIOLOGY, Aug. 1986, p. 455–461
0021-9193/86/080455-07$02.00/0

Vol. 167, No. 2

Gene Order of the TOL Catabolic Plasmid Upper Pathway Operon and Oxidation of Both Toluene and Benzyl Alcohol by the *xylA* Product

S. HARAYAMA,[1]* R. A. LEPPIK,[1]† M. REKIK,[1] N. MERMOD,[1] P. R. LEHRBACH,[1]‡ W. REINEKE,[2] AND K. N. TIMMIS[1]

Department of Medical Biochemistry, University of Geneva, 1211 Geneva 4, Switzerland,[1] and Department of Chemische Mikrobiologie, Bergische Universität, Wuppertal, Federal Republic of Germany

Received 25 November 1985/Accepted 29 April 1986

TOL plasmid pWW0 specifies enzymes for the oxidative catabolism of toluene and xylenes. The upper pathway converts the aromatic hydrocarbons to aromatic carboxylic acids via corresponding alcohols and aldehydes and involves three enzymes: xylene oxygenase, benzyl alcohol dehydrogenase, and benzaldehyde dehydrogenase. The synthesis of these enzymes is positively regulated by the product of *xylR*. Determination of upper pathway enzyme levels in bacteria carrying Tn*5* insertion mutant derivatives of plasmid pWW0-161 has shown that the genes for upper pathway enzymes are organized in an operon with the following order: promoter-*xylC* (benzaldehyde dehydrogenase gene[s])-*xylA* (xylene oxygenase gene[s])-*xylB* (benzyl alcohol dehydrogenase gene). Subcloning of the upper pathway genes in a lambda p_L promoter-containing vector and analysis of their expression in *Escherichia coli* K-12 confirmed this order. Two distinct enzymes were found to attack benzyl alcohol, namely, xylene oxygenase and benzyl alcohol dehydrogenase; and their catalytic activities were additive in the conversion of benzyl alcohol to benzaldehyde. The fact that benzyl alcohol is both a product and a substrate of xylene oxygenase indicates that this enzyme has a relaxed substrate specificity.

TOL plasmid pWW0 of *Pseudomonas putida* mt-2 is a 115-kilobase (kb) transmissible extrachromosomal element which encodes all enzymes required for the oxidative catabolism of a number of aromatic hydrocarbons, including toluene and *m*- and *p*-xylene, via the *meta*-cleavage pathway shown in Fig. 1 (17, 28, 29, 31). Results of analysis of bacteria carrying mutant TOL plasmids suggested that the catabolic genes are organized into two regulatory blocks (30), one of which specifies three enzymes, xylene oxygenase, benzyl alcohol dehydrogenase (BADH), and benzaldehyde dehydrogenase (BZDH), that convert the aromatic hydrocarbons to their corresponding carboxylic acids (upper pathway), whereas the other of which encodes enzymes for the further transformation of the aromatic acids into Krebs cycle intermediates. Subsequent transposon mutagenesis and cloning studies of plasmid pWW0 and its derivatives confirmed that the catabolic genes are organized into two operons which correspond to the two proposed regulatory blocks and which are physically separated on the TOL plasmid by a DNA segment some 10 kb in length (5, 8, 24; this study).

Results of early experiments suggested that expression of the two operons is regulated by the products of two regulatory genes: *xylR* and *xylS*. The *xylR* gene product would regulate both operons, whereas the *xylS* gene product would regulate only the *meta*-cleavage pathway operon (6, 12, 13, 30). More recently the *meta*-cleavage pathway genes and the two regulatory genes *xylS* and *xylR* have been localized precisely on the restriction map of TOL plasmid pWW0 (6, 9a, 11–13; N. Mermod and K. N. Timmis, manuscript in preparation), the promoter regions of these two operons have been identified and sequenced, and their positive regulation at the level of initiation of transcription has been demonstrated (14, 15, 21).

Although the genes, enzymes, and regulation of the *meta*-cleavage pathway have been analyzed in some detail, those of the upper pathway remain poorly characterized. We report here a transposon insertion and cloning analysis of the upper pathway genes of the TOL plasmid. Expression of mutant and hybrid plasmids in *P. putida* and *Escherichia coli* allowed determination of the order and positions of the upper pathway genes and revealed that xylene oxygenase is able to attack not only toluene and xylenes but also their oxidation products, and thus contributes to two of the three catabolic steps of the upper pathway.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacteria and plasmids used in this study are listed in Table 1. Transposon Tn*5* mutant derivatives of TOL plasmid pWW0-161 have been described previously (5, 6). The pGSH2802 plasmid was obtained by inverse transposition of pOT3 (9) to pPL392 (8) and therefore carries the *meta*-cleavage pathway operon plus its regulatory gene *xylS* and contains both the pBR322 and R388 replicons. The latter replicon allows multiplication of this plasmid in *P. putida*. Details of the construction of this plasmid will be reported elsewhere (S. Harayama, M. Rekik, and K. N. Timmis, manuscript in preparation).

Media and culture conditions. L broth (LB) was used as a complete liquid medium, whereas Antibiotic Medium No. 3 (Difco Laboratories, Detroit, Mich.) solidified with 1.5% Bacto-Agar (Difco) was used as a complete solid medium. M9 (19) supplemented with stock salts solution (1) was used

* Corresponding author.
† Present address: Commonwealth Scientific and Industrial Research Organisation, Division of Food Research, Meat Research Laboratory, Cannon Hill, Queensland, Australia.
‡ Present address: Biotechnology Australia Pty. Ltd., Roseville, New South Wales, Australia.

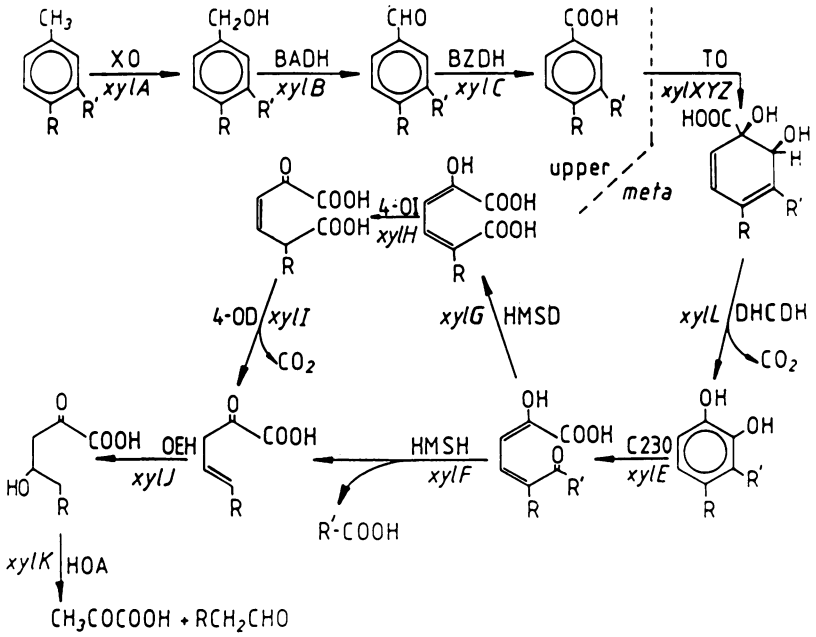

FIG. 1. TOL plasmid-specified pathway for the degradation of toluene and xylenes. Enzyme abbreviations: XO, xylene oxygenase; BADH, benzyl alcohol dehydrogenase; BZDH, benzaldehyde dehydrogenase; TO, toluate dioxygenase; DHCDH, dihydroxycyclohexadiene carboxylate dehydrogenase; C23O, catechol 2,3-dioxygenase; HMSH, hydroxymuconic semialdehyde hydrolase; HMSD, hydroxymuconic semialdehyde dehydrogenase; 4-OI, 4-oxalocrotonate isomerase; 4-OD, 4-oxalocrotonate decarboxylase; OEH, 2-oxopent-4-enoate hydratase; HOA, 2-oxo-4-hydroxy-pentonate aldolase. Initial compounds: R,R'=H, toluene; R=H, R'=CH_3, *m*-xylene; R=CH_3, R'=H, *p*-xylene. *xylA* to *xylZ*, genes for the pathway enzymes (8, 9a, 11, 23, 31). TO is a multicomponent enzyme with structural genes *xylX*, *xylY*, and *xylZ* (9a).

as a minimal liquid or solid (with 1.5% agar) medium. Carbon sources were added to the minimal medium to final concentrations of 10 mM for glucose and acetate and 5 mM for (*m*-methyl)benzyl alcohol, (*m*-methyl)benzaldehyde, *m*-toluate, and benzoate, or supplied as vapor in the case of toluene and *m*-xylene. When used, antibiotics were incorporated into complete medium at the following final concentrations: ampicillin, 100 μg/ml; tetracycline, 10 μg/ml; kanamycin and streptomycin, 25 and 100 μg/ml each for *E. coli* and *P. putida*, respectively. Bacteria were cultivated at 30°C unless otherwise specified.

Enzyme assays. For enzyme assays on *P. putida* cells, a 20- to 40-ml overnight culture in minimal medium plus acetate was diluted into a 200-ml volume of fresh minimal medium plus acetate, which was incubated at 30°C with shaking for 2 h. Where appropriate, (*m*-methyl)benzyl alcohol was added to a final concentration of 5 mM, and the culture was incubated for an additional 4 h. For enzyme assays on *E. coli* K-12 Δ*H*1 Δ*trp* containing a pLV85 derivative, cells on fresh selective plates were inoculated into LB containing ampicillin and cultivated at 30°C to the midexponential phase of growth, then at 42°C for an additional 1.5 to 2.0 h. The cell suspension was then cooled on ice prior to harvesting of bacteria by centrifugation. The pellet obtained was washed with 10 ml of 0.1 M phosphate buffer (pH 7.4).

For whole-cell assays, cells were washed a second time with a 10-ml volume of phosphate buffer and suspended in 5 ml of phosphate buffer, and oxygen consumption was measured at room temperature with an oxygen electrode, in a total volume of 3 ml with either toluene- or *m*-xylene-saturated phosphate buffer (0.1 ml) or (*m*-methyl)benzyl alcohol (final concentration, 50 μM) as substrates. Although substrate-stimulated oxygen consumption was considered to reflect cellular activities which degrade the compound, it was not assumed to imply any specific oxidation mechanism. Toluene- and *m*-xylene- and (*m*-methyl)benzyl alcohol-induced oxygen consumption measured by this assay are therefore termed toluene/xylene oxidase (TXO) and benzyl alcohol oxidase (BAO) activities, respectively.

For cell-free assays, the cell pellet was suspended in 10 ml of 0.1 M phosphate buffer (pH 7.4) containing 10% acetone, and the cells were ruptured by sonication. The cell extract was centrifuged at 20,000 rpm in a Sorvall SS34 rotor for 20 min, and BADH and BZDH activities of the supernatant fluid were assayed as described previously (31), except that cellular protein was determined with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.).

Activities of *E. coli* cells to oxidize toluene were measured by the following method. A tube containing 1 ml of toluene was placed in a 100-ml Erlenmeyer flask containing 2.5 ml of 0.1 M phosphate buffer (pH 7.4), and the mouth of the flask was sealed with Parafilm. About 30 min later, when the air of the flask was saturated with toluene vapor, 2.5 ml of *E. coli* suspension (density, approximately 2.5×10^9 cells per ml, or 5 mg of cellular protein per ml) was quickly added to the buffer in the flask, and the mouth of the flask was again sealed. The flask was incubated with shaking at 30°C for 1 h. The bacterial suspension was then chilled and centrifuged at 20,000 rpm for 2 min in a Sorvall SM24 rotor, and the supernatant fluid obtained was analyzed for concentrations of benzyl alcohol and benzaldehyde as described below. To measure activities of *E. coli* cells to oxidize benzyl alcohol, cells were suspended in 0.1 M phosphate buffer (pH 7.4) containing 1 mM benzyl alcohol and incubated at 30°C with shaking. Fractions (1.2 ml) were removed at 10-min intervals, chilled, and centrifuged for 1.5 min in an Eppendorf model 5414 centrifuge, and the concentration of benzal-

dehyde in the supernatant fluid was determined as described below.

The amount of benzyl alcohol was determined by using the BADH assay. Cell extract (0.5 ml) of *E. coli* K-12 Δ*H*1 Δ*trp*(pGSH2848), which exhibits high BADH activity (see below), was mixed with 1 ml of 0.1 M Tris hydrochloride (pH 8.7) containing 3.3 mM NAD^+. A total of 1 ml of the sample in 0.1 M phosphate buffer (pH 7.4) containing the benzyl alcohol to be assayed was added, and the change in A_{340} was recorded. Oxidation of one molecule of benzyl alcohol by BADH should reduce one NAD^+ molecule; however, this stoichiometry was not observed in the assay, especially at high concentrations of benzyl alcohol, probably as a result of the presence of NADH oxidase in the cell extract. Therefore, the concentration of benzyl alcohol in the sample was determined from a standard curve constructed in each experiment.

To measure the amount of benzaldehyde in 1 ml of sample, 0.1 ml of a freshly prepared solution of 2,4-dinitrophenylhydrazine (0.5 mg/ml in 2 N HCl) was added, and the reaction was allowed to proceed for 15 min at 30°C. A total of 0.4 ml of 5 N NaOH and 0.8 ml of ethanol, both prewarmed to 60°C, were added; and the A_{440} was quickly measured. A standard curve was constructed with a series of mixtures of benzyaldehyde and benzyl alcohol in relative concentrations that varied between 0 μM benzaldehyde plus 1,000 μM benzyl alcohol to 100 μM benzaldehyde plus 900 μM benzyl alcohol.

DNA manipulations. Plasmid DNA was isolated by the methods of Hansen and Olsen (7) and Clewell and Helinski (2) or by the rapid, small-scale procedure of Holmes and Quigley (10). DNA manipulations and analyses were performed as described by Maniatis et al. (19), except for ligation reactions which were carried out by the recommendations of the suppliers of the enzyme.

RESULTS

Phenotypes of *P. putida* containing pWW0-161 and its Tn*5* insertion derivatives. In a previous study (5), a collection of Tn*5* transposon insertion derivatives of the pWW0-161 plasmid were obtained, and the approximate locations of their Tn*5* elements were determined. In the present analysis, the precise map positions of nine of the Tn*5* insertions located within or near the upper pathway genes were determined and correlated with the phenotypes mediated by the mutant plasmids. In Fig. 2 are shown the locations of these elements in relation to restriction sites present in this region of the pWW0 plasmid.

The growth phenotypes of derivatives of *P. putida* PaW85 containing these mutant plasmids were examined by using a variety of upper pathway enzyme substrates (Table 2). Plasmid-free PaW85 grew slowly and poorly on benzaldehyde and benzyl alcohol or did not grow on toluene, *m*-xylene, *m*-methylbenzyl alcohol, or *m*-methylbenzaldehyde. PaW85 apparently can cometabolize the latter two compounds to 3-methylcatechol because production and accumulation of a brown compound was observed when the strain was streaked on plates containing these substrates. Consistent with this observation was the finding that introduction into PaW85 of hybrid plasmid pGSH2802, which carries genes for a complete set of *meta*-cleavage pathway enzymes that transform 3-methylcatechol to tricarboxylic acid cycle intermediates, enabled the organism to grow on *m*-methylbenzyl alcohol and *m*-methylbenzaldehyde, albeit slowly and poorly. Therefore, in addition to the benzoate-degrading enzymes reported previously (25, 28), PaW85 appears to produce enzymes which transform with low efficiency (*m*-methyl)benzyl alcohol to benzoate (*m*-toluate). PaW85 containing pWW0-161 grows well with toluene, *m*-xylene, and (*m*-methyl)benzyl alcohol. However, it grew as poorly with benzaldehyde as did plasmid-free PaW85. The poor growth of PaW85(pWW0-161) with benzaldehyde may be due to poor activity of the latter as an inducer of upper pathway enzyme genes (31), its instability in plates, or both.

Mutant plasmids Tn*5*-118, -51, -86, -60, -74, -54, and -108

TABLE 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Characteristics	Reference or source
E. coli		
K-12 Δ*H*1 Δ*trp*	*lacZ*(Am) Δ(*bio-uvrB*) Δ*trpEA2* λ(*N*am7 *N*am53 *c*I857 Δ*H*1)	27
P. putida		
PaW85	PaW1 cured of pWW0	22
KT2442	Rifampin-resistant derivative of PaW85	5
Plasmids		
pLV85	Derivative of pPLc245 (27) which has multiple cloning sites (*Eco*RI *Sal*I *Bam*HI *Cla*I *Eco*RV *Hpa*I *Hin*dIII; see Fig. 2)	D. O'Connor, unpublished data
pGSH2802	Composite of plasmids R388 and pPL392 (8) which carries complete set of the *meta*-pathway operon genes and regulatory gene *xylS*	Harayama et al., manuscript in preparation
pGSH2873 pGHS2836 pGSH2848 pGSH3033	pLV85 derivatives carrying TOL upper pathway genes (see Fig. 2)	This study
pRME1	pBR322 carrying 1.2-kb *Hae*II segment of Tn*903* conferring resistance to kanamycin (this insertion is flanked by synthetic multiple restriction sites; see Fig. 2	W. Messer, provided by T. Chakrabarty
pWW0-161	Tn*401* (Cb^r) derivative of TOL plasmid pWW0; see Fig. 2 for site of Tn*401* insertion	5
Tn*5*-51 Tn*5*-54 Tn*5*-58 Tn*5*-60 Tn*5*-68 Tn*5*-74 Tn*5*-86 Tn*5*-108 Tn*5*-118	Tn*5* (Km^r) insertion derivatives of pWW0-161 (see Fig. 2)	5

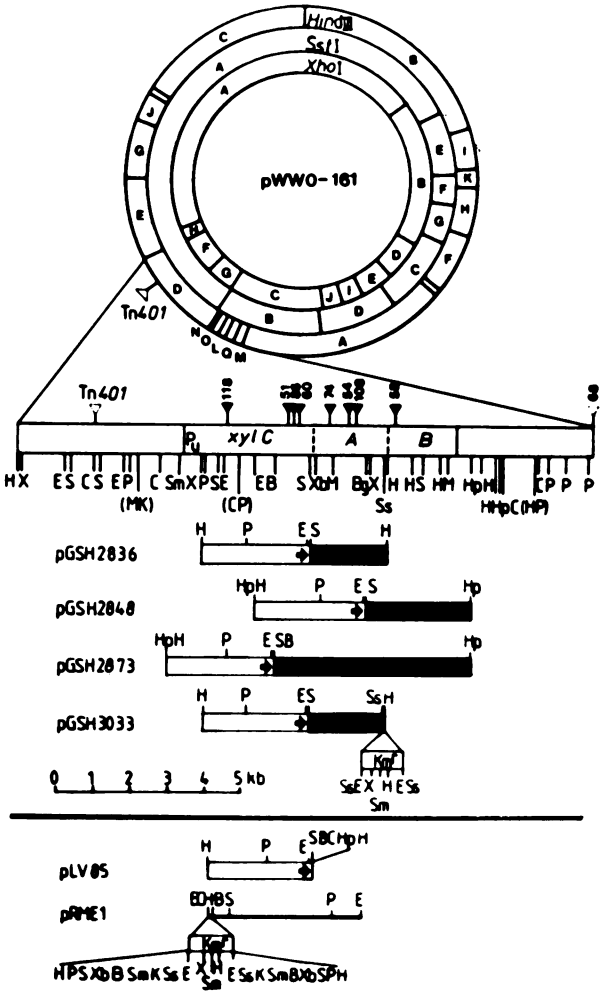

FIG. 2. Physical and genetic maps of the upper pathway region of TOL plasmid pWW0-161 and its subclones. The locations of *Hin*dIII, *Sst*I, *Xho*I, *Eco*RI, and *Bam*HI sites of pWW0 and the site of insertion of Tn*401* in pWW0-161 are taken from previously published data (3, 5, 8, 11–13, 18, 24). Previously reported Tn*5* insertions in pWW0-161 (5) were remapped in this study relative to known restriction sites in Tn*5* (16). Filled symbols indicate mutations that inactivate catabolic functions, whereas open symbols indicate insertions that do not affect such functions. The detailed restriction map of the TOL segment encoding the upper pathway operon region was generated in this study. pGSH series hybrid plasmids contain upper pathway gene segments cloned in lambda p_L promoter vector pLV85 (orientation of p_L indicated by arrows). Closed and open boxes represent TOL DNA segments and pLV85 plasmid, respectively. Plasmid pGSH3033 was constructed by inserting into the *Sst*I site of pGSH2836 a 1.2-kb *Sst*I fragment of plasmid pRME1 containing the structural gene for kanamycin resistance. This segment was originally isolated as a *Hae*II fragment of Tn*903* that was cloned into a polylinker sequence. The structures of pLV85 and pRME1 are shown. The closed bar of pRME1 is pBR322 DNA. Restriction sites abbreviations: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII, Hp, *Hpa*I; K, *Kpn*I; M, *Mlu*I; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; Ss, *Sst*I; X, *Xho*I, Xb, *Xba*I.

did not endow on PaW85 the ability to utilize as growth substrates toluene or *m*-xylene, nor did they stimulate its growth on (*m*-methyl)benzyl alcohol. PaW85 containing mutant plasmid Tn*5*-58 grew on (*m*-methyl)benzyl alcohol at a rate slightly slower than that of PaW85(pWW0-161), and poorly with toluene and *m*-xylene. The growth properties of PaW85(Tn*5*-68) were the same as those of PaW85(pWW0-161).

The levels of upper pathway enzymes in PaW85 containing mutant plasmids are presented in Table 2. As can be seen from the data in Fig. 2 and Table 2, bacteria carrying mutant plasmids Tn*5*-118, -51, -86, and -60 (i.e., containing Tn*5* insertions on the left side of the map as drawn) lacked all upper pathway enzymes, whereas bacteria carrying mutant plasmids Tn*5*-74, -54, and -108 (i.e., containing Tn*5* insertions toward the right side of the map) had high levels of BZDH but not other enzymes. Bacteria carrying mutant plasmid Tn*5*-58 exhibited inducible BZDH and BAO activities but not TXO and BADH activities. The Tn*5* element at the right end of the gene cluster (i.e., in Tn*5*-68) did not affect expression of any of the catabolic genes.

From these results it can be concluded that the structural gene(s) for BZDH (*xylC*) is located between the operon promoter (14) and the site of the Tn*5* insertion in mutant plasmid Tn*5*-74. The structural gene for BADH (*xylB*) has been previously localized on the DNA segment containing the *Hin*dIII NOLQ fragments (11). Our observation that bacteria carrying Tn*5*-58, in which the Tn*5* element is located in *Hin*dIII fragment N, lacked BADH activity is consistent with this conclusion. From these results it would seem that *xylA*, the structural gene for XO, is located between *xylC* and *xylB*. However, the observation that PaW85(Tn*5*-58) exhibited inducible BAO activity and grew on (*m*-methyl)benzyl alcohol suggests that this region specifies a second TOL enzyme (i.e., in addition to BADH) that is able to metabolize (*m*-methyl)benzyl alcohol.

Cloning and expression of upper pathway enzyme genes in *E. coli*. To define more precisely the organization of genes of upper pathway enzymes, various DNA fragments were cloned in the lambda p_L promoter-containing vector pLV85, and their expression was examined in *E. coli*. Plasmids pGSH2873, -2836 and -2848 carry 5.2-kb *Bam*HI-*Hpa*I, 2.1-kb *Sal*I-*Hin*dIII, and 2.8-kb *Bgl*II-*Hpa*I segments of TOL plasmid, respectively (Fig. 2), and were maintained in *E. coli* K-12 Δ*H*1 Δ*trp*, which contains a temperature-sensitive lambda *c*I857 repressor gene. Cloned genes were induced by cultivation of host bacteria at 42°C; and activities of TXO, BAO, and BADH were measured. Cells containing pGSH2873 exhibited TXO, BAO, and BADH activities; those containing pGSH2836 exhibited BAO and TXO but not BADH activities; and pGSH2848 showed BAO and BADH but not TXO activities (Table 3).

Transformation of toluene and benzyl alcohol by induced cells of *E. coli* containing these plasmids was also examined. Cells containing pGSH2836 which are TXO^+, BAO^+, and $BADH^-$ transformed toluene to benzyl alcohol and benzyl alcohol to benzaldehyde (Table 3). This result shows that the 2.1-kb *Sal*I-*Hin*dIII segment specifies two enzymatic activities, one for the oxidation of toluene to benzyl alcohol and one for the oxidation of benzyl alcohol to benzaldehyde, and that benzyl alcohol-oxidizing activity encoded by this segment is distinct from that of BADH. In contrast, cells containing the TXO^- BAO^+ $BADH^+$ plasmid, pGSH2848, metabolized benzyl alcohol to benzaldehyde but not toluene to benzyl alcohol. Cells containing pGSH2873 (TXO^+ BAO^+ $BADH^+$) transformed, as expected, toluene to benzyl alcohol and benzyl alcohol to benzaldehyde. Interestingly, cells containing pGSH2873 produced 10 times more benzaldehyde from toluene than did those containing pGSH2836. Plasmid pGSH3033, which is a derivative of pGSH2836 that contains a 1.4-kb *Hae*II fragment of Tn*903* (26) inserted at its unique

TABLE 2. Growth phenotypes and enzyme levels of *P. putida* KT2442 derivatives containing pWW0-161 or its Tn*5* insertion mutants[a]

Plasmids	Growth substrate[b]						Enzyme activity[c]			
	T	X	Bl	mBl	Bd	mBd	BZDH	BAO	BADH	TXO
None	−	−	±	−	±	−	*	*	*	*
pGSH2802	−	−	±	±	±	±	*	*	*	*
pWW0-161	+	+	+	+	±	±	88 (22)	86 (13)	56 (4)	22 (4)
Tn*5*-118	−	−	±	±	±	±	5	<3	2	<3
Tn*5*-51	−	−	±	±	±	±	3	8	4	<3
Tn*5*-86	−	−	±	±	±	±	3	*	2	*
Tn*5*-60	−	−	±	±	±	±	3	5	2	4
Tn*5*-74	−	−	±	±	±	±	79	<3	2	<3
Tn*5*-54	−	−	±	±	±	±	66	8	2	*
Tn*5*-108	−	−	±	±	±	±	100 (8)	3 (2)	4 (6)	8
Tn*5*-58	±	±	+	+	±	±	140	52 (9)	3	8
Tn*5*-68	+	+	+	+	±	±	84	36	61	30

[a] Abbreviations: T, toluene; X, *m*-xylene; Bl, benzyl alcohol; mBl, *m*-methylbenzyl alcohol; Bd, benzaldehyde; mBd, *m*-methylbenzaldehyde; BZDH, benzaldehyde dehydrogenase; BAO benzyl alcohol oxidase; BADH, benzyl alcohol dehydrogenase; TXO, toluene xylene oxidase.
[b] Symbols: −, no growth after 6 days at 30°C; ±, colonies visible after 3 to 5 days at 30°C; +, colonies visible after 1 to 2 days and large after 4 days.
[c] BZDH and BADH activities are expressed as nanomoles of NAD^+ reduced per minute per milligram of protein, whereas BAO and TXO activities are expressed as nanomoles of oxygen consumed per minute per milligram of protein. Parentheses indicate noninduced level of enzyme activities, whereas an asterisk indicates not tested.

*Sst*I site, does not specify any activity that transforms toluene or benzyl alcohol (Table 3).

DISCUSSION

In this study we localized TOL plasmid genes of enzymes that catalyze the transformation of toluene and xylenes to corresponding aromatic carboxylic acids, and examined the synthesis of these enzymes in bacteria carrying mutant TOL plasmids and hybrid plasmids carrying segments of TOL.

The structural gene(s) for BZDH (*xylC*) was shown to be located upstream of insertion Tn*5*-74, which is situated some 3 kb downstream of the upper pathway operon promoter that has been identified and sequenced by Inouye et al. (14).

Our subcloning experiments located *xylB*, the structural gene for BADH, to a 2.8-kb *Bgl*II-*Hpa*I fragment. Subcloning in *E. coli* of a 2.1-kb *Sal*I-*Hin*dIII segment of TOL DNA, located downstream of *xylC* and upstream of *xylB*, demonstrated that it specifies two enzymatic activities that transform toluene to benzyl alcohol and benzyl alcohol to benzaldehyde. These two activities were simultaneously inactivated by insertion of a segment of Tn*903* at the unique *Sst*I site that is located only 100 base pairs upstream of the promoter-distal *Hin*dIII end of the 2.1-kb segment. It is very likely, therefore, that only one gene is affected by the insertion, and it follows that one polypeptide specified by the promoter-distal portion of the 2.1-kb segment is required for both activities. This same polypeptide is also involved in a third activity, namely, the oxidation of indole to indoxyl (20). Thus, XO seems to have a particularly relaxed substrate specificity and catalyzes the oxidation of toluene and xylene, (*m*-methyl)benzyl alcohol, and indole. An alternative interpretation of the phenotype of the cloned 2.1-kb segment, namely, that it encodes three different multicomponent enzymes which share a common subunit, would seem to be unlikely because of the limited coding capacity of the fragment (preliminary experiments have thus far identified two polypeptides with molecular weights of 35,000 and 40,000; S. Harayama, unpublished data).

At first sight, the interpretation that XO is encoded in the 2.1-kb segment seems to be contradicted by the observation that Tn*5*-58, which is located downstream of the 2.1-kb segment, affected TXO activity (Table 2). This discrepancy is, however, due to the assay method of TXO which measures multiple oxygen-consuming reactions resulting from successive oxidation of toluene-xylene. In Tn*5*-58 bacteria, in which BADH is defective, toluene-xylene is oxidized to (*m*-methyl)benzyl alcohol, although further oxidation may take place only slowly, as is the case in TXO^+ $BADH^-$ *E. coli*(pGSH2836) bacteria (Table 3). The oxygen consumption cascade is therefore not fully induced by the addition of toluene-xylene to Tn*5*-58 bacteria; and TXO levels measured are low, even though it contains active TXO. The slow growth rate of this strain with toluene-xylene is similarly explained by the slow conversion of (*m*-methyl)benzyl alcohol to the aldehyde as a result of the defect in BADH. On the other hand, it grew well with 5 mM (*m*-methyl)benzyl alcohol and exhibited quasinormal (*m*-methyl)benzyl alcohol-stimulated oxygen consumption. We interpret these re-

TABLE 3. Oxidation of toluene and benzyl alcohol by washed cells of *E. coli* containing cloned TOL upper pathwayt enzyme genes[a]

Plasmid	Enzyme activity[b]			Rate of oxidation product formation[c]			
	TXO	BAO	BADH	TOL→ Bl	TOL→ Bd	Total	Bl→ Bd
pGSH2836	2.8	8.4	0	1.9	0.5	2.4	14
pGSH2848	<0.4	3.1	83	0	0	0	40
pGSH2873	3.0	14.6	83	0.6	5.3	5.9	88
pGSH3033	<0.4	<0.4	0	0	0	0	0

[a] Host strain was K-12 Δ*H*1 Δ*trp*. Abbreviations and enzymatic activities are as described in footnote *a* of Table 2.
[b] Enzymatic activities are corrected for values obtained with K-12 Δ*H*1 Δ*trp*(pLV85), on the assumption that specific activities in this strain are zero.
[c] Rates of product formation are expressed as nanomoles of product formed per minute per mg milligram of protein (TOL→ Bl and TOL→ Bd, conversion from toluene to benzyl alcohol and benzaldehyde, respectively; total, total activities of toluene degradation; Bl→ Bd, conversion of benzyl alcohol to benzaldehyde). In experiments with toluene as the substrate, the rate of product formation was calculated from the amount of product formed in 60 min divided by 60, whereas the formation of benzaldehyde from benzyl alcohol was calculated from initial rates obtained in time course experiments.

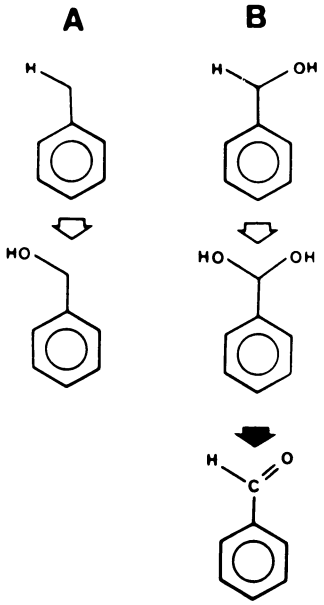

FIG. 3. Possible mechanism of action of xylene oxygenase. Xylene oxygenase may recognize the aromatic ring and part of the structure of the side chain, —C—H, and catalyze replacement of the hydrogen by OH (open arrow). If the substrate is toluene, the product is benzyl alcohol (A); whereas if the substrate is benzyl alcohol, its product is phenyl methane diol (B) which will spontaneously dehydrate to form benzaldehyde (closed arrow).

sults to imply that although the efficiency of XO-catalyzed oxidation of (*m*-methyl)benzyl alcohol is low at those concentrations produced by oxidation of toluene-xylene delivered to bacteria as vapor, it can be much higher at the intracellular concentrations achieved by the exogenous supply of this substrate at a concentration of 5 mM. This deduction, inferred from the growth phenotype of Tn*5*-58, was confirmed in an independent experiment in which significant conversion of benzyl alcohol to benzaldehyde and significant BAO activity were observed in *E. coli* cells harboring the XO^+ $BADH^-$ plasmid pGSH2836 when 5 mM benzyl alcohol was exogenously supplied (Table 3). Therefore, we attributed BAO activity observed in *P. putida* containing Tn*5*-58 and in *E. coli* containing pGSH2836 to that of XO. We interpreted, however, the weak BAO activity observed in *E. coli* bacteria carrying a 2.8-kb *Bgl*II-*Hpa*I fragment (pGSH2848; Table 3) as a reflection of BADH, which produces NADH by oxidizing benzyl alcohol and, in turn, which may stimulate cellular respiration.

In conclusion, although XO can oxidize (*m*-methyl)benzyl alcohol, the principal biological role of this enzyme is to transform toluene-xylene to (*m*-methyl)benzyl alcohol, which, in turn, is mainly transformed by BADH.

Although the mechanism of action of this relaxed substrate specificity of XO needs to be defined by biochemical studies, the structural similarity of substrates which it can transform indicates one possible catalytic sequence. This would be that the enzyme recognizes the aromatic ring and the H—C bond of the side chain shown in Fig. 3. The reaction might be initiated by removal of one hydrogen atom from the side chain, followed by the addition of a hydroxyl group. Whereas the product of oxidation of toluene (*m*-xylene) is (*m*-methyl)benzyl alcohol, the product of oxidation of (*m*-methyl)benzyl alcohol would have a side chain carbon atom carrying two hydroxyl groups. This latter structure would be unstable and would spontaneously dehydrate to form (*m*-methyl)benzaldehyde. The oxidation product of indole would be indoxyl which, in the presence of oxygen, would conjugate to form indigo (4).

In conclusion, the genes of the three upper pathway enzymes of the TOL catabolic plasmid are clustered in a 7-kb DNA segment and are arranged in the following order: promoter-*xylC-xylA-xylB*. This segment may additionally contain genes for other proteins involved in the catabolism of upper pathway substrates, such as substrate transport proteins or accessory electron transfer components; this possibility is currently being investigated.

ACKNOWLEDGMENTS

We thank D. O'Connor and T. Chakraborty for gifts of plasmids and F. Rey for typing the manuscript.

This work was supported in part by grants from the Deutsche Forschungsgemeinschaft (to W.R.), the Australia Meat Research Council (to K.N.T.), and Transgène S. A. (to K.N.T.).

LITERATURE CITED

1. **Bauchop, T., and S. R. Eldsen.** 1960. The growth of microorganisms in relation to their energy supply. J. Gen. Microbiol. **23**:457–469.
2. **Clewell, D. B., and D. R. Helinski.** 1969. Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. USA **62**:1159–1166.
3. **Downing, R., and P. Broda.** 1979. A cleavage map of the TOL plasmid of *Pseudomonas putida* mt-2. Mol. Gen. Genet. **177**:189–191.
4. **Ensley, D. B., B. J. Ratzkin, T. D. Osslund, M. J. Simon, L. P. Wackett, and D. T. Gibson.** 1983. Expression of naphthalene oxidation genes in *Escherichia coli* results in the biosynthesis of indigo. Science **222**:167–169.
5. **Franklin, F. C. H., M. Bagdasarian, M. M. Bagdasarian, and K. N. Timmis.** 1981. Molecular and functional analysis of the TOL plasmid pWW0 from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring *meta* cleavage pathway. Proc. Natl. Acad. Sci. USA **78**:7458–7462.
6. **Franklin, F. C. H., P. R. Lehrbach, R. Lurz, B. Rueckert, M. Bagdasarian, and K. N. Timmis.** 1983. Localization and functional analysis of transposon mutations in regulatory genes of the TOL catabolic pathway. J. Bacteriol. **154**:676–685.
7. **Hansen, J. B., and R. H. Olsen.** 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol. **135**:227–238.
8. **Harayama, S., P. R. Lehrbach, and K. N. Timmis.** 1984. Transposon mutagenesis analysis of *meta*-cleavage pathway operon genes of the TOL plasmid of *Pseudomonas putida* mt-2. J. Bacteriol. **160**:251–255.
9. **Harayama, S., T. Oguchi, and T. Iino.** 1984. Does Tn*10* transpose via the cointegrate molecule? Mol. Gen. Genet. **194**: 444–450.
9a. **Harayama, S., M. Rekik, and K. N. Timmis.** 1986. Genetic analysis of a relaxed substrate specificity aromatic ring dioxygenase, toluate 1,2-dioxygenase, encoded by TOL plasmid pWW0 of *Pseudomonas putida*. Mol. Gen. Genet. **202**:226–234.
10. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**: 193–197.
11. **Inouye, S., A. Nakazawa, and T. Nakazawa.** 1981. Molecular cloning of TOL genes *xylB* and *xylE* in *Escherichia coli*. J. Bacteriol. **145**:1137–1143.
12. **Inouye, S., A. Nakazawa, and T. Nakazawa.** 1981. Molecular cloning of genes *xylS* of the TOL plasmid: evidence for positive regulation of *xylDEGF* operon by *xylS*. J. Bacteriol. **148**: 413–418.
13. **Inouye, S., A. Nakazawa, and T. Nakazawa.** 1983. Molecular cloning of regulatory gene *xylR* and operator-promoter regions of the *xylABC* and *xylDEGF* operons of the TOL plasmid. J.

Bacteriol. **155**:1192–1199.
14. **Inouye, S., A. Nakazawa, and T. Nakazawa.** 1984. Nucleotide sequence surrounding the transcription initiation site of *xylABC* operon on TOL plasmid of *Pseudomonas putida*. Proc. Natl. Acad. Sci. USA **81**:1688–1691.
15. **Inouye, S., A. Nakazawa, and T. Nakazawa.** 1984. Nucleotide sequence of the promoter region of the *xylDEGF* operon on TOL plasmid of *Pseudomonas putida*. Gene **29**:323–330.
16. **Jorgensen, R. A., S. J. Rothstein, and W. S. Reznikoff.** 1979. A restriction enzyme cleavage map of Tn*5* and location of a region encoding neomycin resistance. Mol. Gen. Genet. **177**:65–72.
17. **Kunz, D. A., and P. J. Chapman.** 1981. Catabolism of Pseudocumene and 3-ethyltoluene by *Pseudomonas putida* (*arvilla*) mt-2: evidence for new functions of the TOL (pWW0) plasmid. J. Bacteriol. **146**:179–191.
18. **Lehrbach, P. R., J. Ward, P. Meulien, and P. Broda.** 1982. Physical mapping of TOL plasmids pWW0 and pND2 and various R plasmid-TOL derivatives from *Pseudomonas* spp. J. Bacteriol. **152**:1280–1283.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Mermod, N., S. Harayama, and K. N. Timmis.** 1986. New route to bacterial production of indigo. BioTechnology **4**:321–324.
21. **Mermod, N., P. R. Lehrbach, W. Reineke, and K. N. Timmis.** 1984. Transcription of the TOL plasmid toluate catabolic pathway operon of *Pseudomonas putida* is determined by a pair of co-ordinately and positively regulated promoters. EMBO J. **3**:2461–2466.
22. **Meulien, P., and P. Broda.** 1982. Identification of chromosomally integrated TOL DNA in cured derivatives of *Pseudomonas putida* PAW1. J. Bacteriol. **152**:911–914.
23. **Murray, K., D. J. Dugglebey, J. M. Sala-trepat, and P. Williams.** 1972. The metabolism of benzoate and methylbenzoates via the *meta*-cleavage pathway by *Pseudomonas arvilla* mt-2. Eur. J. Biochem. **28**:301–310.
24. **Nakazawa, T., S. Inouye, and A. Nakazawa.** 1980. Physical and functional mapping of RP4-TOL plasmid recombination: analysis of insertion and deletion mutants. J. Bacteriol. **144**:222–231.
25. **Nakazawa, T., and T. Yokota.** 1973. Benzoate metabolism in *Pseudomonas putida* (*arvilla*) mt-2: demonstration of two benzoate pathways. J. Bacteriol. **115**:262–267.
26. **Oka, A., H. Sugisaki, and M. Takanami.** 1980. Nucleotide sequence of the kanamycin resistance transposon Tn*903*. J. Mol. Biol. **147**:217–226.
27. **Remaut, E., P. Stanssens, and W. Fiers.** 1983. Inducible high level synthesis of mature human fibroblast interferon in *Escherichia coli*. Nucleic Acids Res. **11**:4677–4688.
28. **Williams, P. A., and K. Murray.** 1974. Metabolism of benzoate and the methyl benzoates by *Pseudomonas putida* (*arvilla*) mt-2: evidence for the existence of a TOL plasmid. J. Bacteriol. **120**:416–423.
29. **Wong, C. L., and N. W. Dunn.** 1974. Transmissible plasmid coding for the degradation of benzoate and *m*-toluate in *Pseudomonas arvilla* mt-2. Genet. Res. **23**:227–232.
30. **Worsey, M. J., F. C. H. Franklin, and P. A. Williams.** 1978. Regulation of the degradative pathway enzymes coded for by the TOL plasmid (pWW0) from *Pseudomonas putida* mt-2. J. Bacteriol. **134**:757–764.
31. **Worsey, M. J., and P. A. Williams.** 1975. Metabolism of toluene and xylenes by *Pseudomonas putida* (*arvilla*) mt-2. Evidence for a new function of the TOL plasmid. J. Bacteriol. **124**:7–13.